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Dear Dr. Kuniko Yasue,

Kindly please lest us to submit our manuscript entitled " Orally Administered D-Aspartate Depresses Rectal Temperature and Alters Plasma Triacylglycerol and Glucose Concentrations in Broiler Chicks to Journal of Poultry Science".

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Orally Administered D-Aspartate Depresses Rectal Temperature and Alters Plasma Triacylglycerol and Glucose Concentrations in Broiler Chicks

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Running head: D-Aspartate alters rectal temperature and plasma metabolites

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Abstract

Both L-aspartate (L-Asp) and D-aspartate (D-Asp) play important physiological roles in animals. However, there are very few studies available in birds and no reports have been found in broilers. Broilers are very important commercial birds for meat production, so effects of L- or D-Asp in broilers would provide new physiological insight of this strain. Therefore, the purpose of this study was to determine the effect of oral administration of L- or D-Asp on food intake, body temperature and some plasma metabolites in broiler chicks. Broiler chicks were orally administered with different doses (0, 3.75, 7.5 and 15 mmol/kg body weight) of L- or D-Asp. We confirmed that oral administration of both L- and D-Asp did not alter food intake. However, D-Asp, but not L-Asp, dose-dependently decreased the rectal temperature in chicks. It was also found that D-Asp increased plasma glucose and decreased triacylglycerol concentrations. The changes in plasma metabolites further indicate that D-Asp treatment modulates the energy metabolism in broiler chicks. In conclusion, D-Asp may be a beneficial nutrients for broilers, since orally administered D-Asp lowered body temperature without reducing food intake.

Key words: L-Aspartate; D-aspartate; body temperature; plasma metabolites, broiler chicks

Introduction

High ambient temperature (HT) induced heat-stress is a serious concern over the globe. The Intergovernmental Panel on Climate Change (IPCC) (2015) reported that the global surface temperature is increasing in this century. Therefore, probably summer time is becoming more unbearable in many tropical and subtropical countries. It is well known that summer heat stress is causing a great economic loss in commercial poultry sector. Donkoh (1989) reported that continuous exposure of broilers to HT markedly affected their performance and physiological functions. HT induces quick increment of deep body temperature even in chicks (Chowdhury *et al.,* 2012) and causes oxidative stress (Chowdhury *et al.,* 2014). To cool down the temperature in the poultry house using fossil energy leads further increase in the environmental temperature. Therefore, it is very important to find out other strategies to control the body temperature in the chicken. For instance, suitable nutrients or drugs to reduce high body temperature when exposed to HT should be clarified. Nutrients, especially amino acids, are widely applied as anti-stress agents with regard to psychological and physiological stress (Yamane *et al.,* 2009; Hamasu *et al.,* 2010; Erwan *et al.,* 2012, 2014b). Amino acid, in particular essential amino acids, supplementation has been performed in order to overcome heat-stress problems in birds (Mendes et al., 1997; Brake *et al.,* 1998; Willemsen *et al.,* 2011; Dai *et al.,* 2012). However, not only essential amino acids, but also non-essential amino acids may be useful to mitigate heat-stress problems. Recently, Chowdhury *et al.* (2015) showed that a non-essential amino acid, L-citrulline, reduced body temperature in young chicks and suggested its possibility to use as a hypothermic agent under heat-stress in chickens.

Amino acids can exist in two-mirror-image form (L- or D-form) termed enantiomers. Aspartate (Asp), which is a non-essential amino acid, plays important roles as a constituent of protein synthesis, a precursor of specific neurotransmitters and physiological modulators (Spinelli *et al.,* 2006; Schell *et al.,* 1997; Errico *et al.,* 2009).

L- and D-Asp have been shown to occur in various brain regions of chickens (Neidle and Dunlop, 1990) and pigeons (Kera *et al.,* 1996). As far as the physiological roles in correlation with D-Asp in mammals and birds are concerned, important biological functions of this amino acid are now postulated (Topo *et al.,* 2010). Though some evidence to date suggests that D-Asp plays important physiological roles in adult organisms, but little is known about the function of D-Asp during an early development when its level is physiologically high (Homma, 2007; Errico *et al.,* 2008). Therefore, it is worthy to use growing animals to study the effect of D-Asp, so neonatal chicks were used in this study.

It has been reported that the injection of D-Asp released the antidiuretic hormone arginine vasopressin (AVP) in rats (Koyuncuoğlu *et al.,* 1984). Studies in rats have further shown that D-Asp not only lowered body temperature after oral administration (Koyuncuoğlu *et al.,* 1982a; Koyuncuoğlu and Berkman, 1982), but also decreased plasma triacylglycerol (TG) (Koyuncuoğlu *et al.,* 1982b). We have previously shown that intracerebroventicular (i.c.v.) injection of either L- or D-Asp clearly attenuated stress responses in layer chicks (Yamane *et al.,* 2009; Erwan *et al.,* 2012, 2014b) and oral administration of D-Asp decreased food intake, body temperature and altered some plasma metabolites in layer chicks (Erwan *et al.,* 2013a, 2014a). Hence, L- or D-Asp may act as a modulator of important physiological functions in chicks. However, some physiological functions are different between layer and broiler chicks. Tachibana *et al.* (2001) reported that agouti-related peptide, a potent stimulator of food intake (Rossi *et al.,* 1998), stimulated food intake only in layer type chicks but not broiler chicks. Therefore, it is important to know whether D-Asp can regulate body temperature and influence food intake in broilers. The purpose of this study was to determine the effect of L- or D-Asp on food intake, body temperature and plasma metabolites in broiler chicks.

Materials and Methods

Animals and Drugs

One-day-old un-sexed-broiler chicks (Cobb) (*Gallus gallus domesticus*) were purchased from a local hatchery (Charoen Pokphand Jaya Farm Ltd, Pekanbaru, Indonesia) and housed in a wooden cage (50 × 35 × 33 cm) in a group (25 birds) at a constant temperature of 30 ± 1°C with continuous lighting. Food (Commercial starter diet, 311-VIVO, Pokphand Tbk, Medan, Indonesia) and water were provided *ad libitum*. One day before the experiment (4 days old), thirty two chicks were reared individually and assigned for treatment and control groups on the basis of their body weight in order to produce uniform groups in both Experiments 1 and 2. This study was performed in accordance with the guidelines for animal experiments carried out at the Faculty of Agriculture and Animal Science, State Islamic University of Sultan Syarif Kasim Riau, Pekanbaru, Indonesia.

L- and D-Asp were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Administration of L- or D-Asp

Following an acclimatization period with individual rearing for 24 hr, chicks were randomly selected and divided into four groups, each group consisting of 8 chicks. The birds were provided with *ad libitum* access to the diet during the whole experimental period. On the day of the experiment, each chick (5 days old) was orally administered either a solution of L-Asp (Experiment 1) or D-Asp (Experiment 2) for the treatment groups, or distilled water (DW) for the control group, via an elastic plastic needle on a small syringe. Based on our recent report on L- or D-Asp in layer chicks (Erwan *et al.,* 2013a, 2014a), oral administration of L- or D-Asp in Experiments 1 and 2, has been designed to use 3.75, 7.5 and 15.0 mmol/ kg body weight as the low, medium and high doses, respectively.

At 120 min after administration of L- or D-Asp, all birds in each group were sacrificed and their bloods were collected for analysis of plasma metabolites.

Measurement of Food Intake and Rectal Temperature

Food intake (at 30, 60 and 120 min) was determined by measuring the reduction in the amount of food consumed from a pre-weighed feeder. The rectal temperature of chicks was also measured at 30-, 60- and 120-min of the experimental time with a digital thermometer with an accuracy of ± 0.1°C (Thermalert TH-5, Physitemp Instruments Inc., USA) by inserting the thermistor probe into the cloaca to a depth of 2 cm.

Analysis of Plasma Metabolites

Blood was collected into heparinized eppendorf tubes and centrifuged at 5,000 rpm for 15 min. Plasma was stored at −20°C until assay. The plasma metabolites (TG, glucose, total cholesterol (TCHO) and total protein (TP)) were measured with Microlab 300 (Vital Scientific, Netherland) as per the manufacturer's instructions. All the samples were assayed together and in a random sequence for each metabolite.

Statistical Analysis

For the rectal temperature, a repeated-measures two-way analysis of variance (ANOVA) was applied. Plasma metabolites were statistically analyzed by one-way ANOVA and regression equations. The Tukey test was done as a post hoc test. Significant differences were denoted as $P<0.05$. Values were presented as means ± S.E.M. Statistical analysis was carried out using the commercially available package StatView (Version 5, SAS Institute, Cary, USA, 1998). All data in each group were first subjected to a Thompson rejection test to eliminate outliers ($P<0.05$), and the remaining data were used for the analysis among groups.

Results

Experiment 1: Effects of L-Asp on Food Intake, Rectal Temperature and Plasma Metabolites

Oral administration of several doses of L-Asp did not alter food intake in chicks (*data not shown*). As shown in Fig. 1, rectal temperatures of chicks were not changed significantly by the oral administration of L-Asp ($F(3, 21) = 1.92$, $P>0.05$). Table 1 shows the effect of oral administration of several doses of L-Asp on the concentration of plasma metabolites. There was no significant effect of L-Asp on the concentration of plasma metabolites.

Experiment 2: Effects of D-Asp on Food Intake, Rectal Temperature and Plasma Metabolites

Oral administration of several doses of D-Asp did not significantly alter food intake in chicks (*data not shown*). However, D-Asp significantly declined rectal temperatures in broiler chicks ($F(3, 20) = 5.81$, $P<0.005$) as shown in Fig. 2. D-Asp further showed a significant effect of time ($F(3, 2) = 25.09$, $P<0.0001$) and an interaction between the dose and time ($F(6, 40) = 4.70$, $P<0.005$) on declining body temperature, implying that the effect of D-Asp was more prominent with the progress of time and the low dose of the treatment caused more reduction in body temperature. Table 2 shows the changes in plasma metabolites due to the oral administration of D-Asp. A significant ($P<0.005$) negative correlation was detected between the administered doses of D-Asp and the plasma concentration of TG (143.4 [SE 10.4] – 4.1 [SE 1.2] X, $R^2 = 0.295$). Plasma TG concentration significant decreased by 15 mmol/kg body weight D-Asp, compared with the control ($F(3, 25) = 6.51$ $P<0.005$). On the other hand, a significant ($P<0.005$) positive correlation was observed between the administered doses of D-Asp and the plasma concentration of glucose (124.6 [SE 2.2] +

1.0 [SE 0.3] X, $R^2 = 0.348$). Plasma glucose concentration was significant increased by 15 mmol/ kg body weight D-Asp, compared with the control ($F(3, 23) = 5.00$ $P<0.01$). The values of TP and TCHO showed no significant changes in any of the treated groups.

Discussion

We confirmed that oral administration of L-or D-Asp did not affect food intake in broiler chicks. Our current findings on L-Asp concerning food intake in broiler chicks is in accordance with other reports where it was shown that either peripheral or central administration of L-Asp did not influence food intake in layer chicks (Maruyama *et al.,* 1972; Bungo *et al.,* 2002; Erwan *et al.,* 2013a). However, the effect of D-Asp on food intake differed with our recent study (Erwan *et al.,* 2013a), since food intake was significantly decreased when layer chicks were orally administrated with D-Asp. The reason for these discrepancies on food intake behavior due to D-Asp is unknown. However, food intake and growth rate is 1.5 to 2-fold greater in broilers than in layers at 2 or 3 days old (Masic *et al.,* 1974; Mahagna and Nir, 1996). Strain specific feeding regulation and variation in metabolic rate may cause the different responses for L- and D-Asp. Furthermore, it has been well documented that L- or D-Asp has a function as neurotransmitters to influence the central nervous system to regulate food intake in rats. L-Asp is not only concentrated in nerve endings (Gundersen *et al.,* 1991), but is also found localized and accumulated in common synaptic vesicles (Gundersen *et al.,* 1998; Fleck *et al.,* 2001). Thus, it could be speculated that the administered dose of D-Asp might not have influenced physiological actions on neurocircuits in controlling feeding behavior in broiler chicks.

We also demonstrated here that L-Asp did not influence body temperature in broiler chicks. The result was accordance with the previous findings that oral administration of L-Asp did not decrease rectal temperature in rats (Koyuncuoğlu *et al.,*

1982a; Koyuncuoğlu and Berkman, 1982 and in layer chicks (Erwan *et al.,* 2014a]. Interestingly, we have shown that when L-Asp was chemically conjugated with lauric acid, a medium-chain fatty acid, then it enabled decreasing body temperature in layer chicks (Erwan *et al.,* 2013b). Thus, it could be suggested that the chemical structure of L-Asp is not designed to influence body temperature but esterified L-Asp may have that potential.

When focusing on lowered body temperature due to the oral administered D-Asp, there was consistency with previous research in rats and layer chicks (Koyuncuoğlu *et al.,* 1982a; Koyuncuoğlu and Berkman, 1982; Erwan *et al.,* 2014a). In addition, Koyuncuoğlu *et al.* (1982b) demonstrated that rectal temperature significantly decreased when rats received D-Asp or D- plus L-Asp in a 1:1 ratio. Since the decreased body temperature by D-amino acids was antagonized by naloxone, an opioid antagonist (Koyuncuoğlu *et al.,* 1982a), the decrease in rectal temperature might be caused by opioid system. Previous reports demonstrated that the injection of D-Asp increased AVP in rats (Koyuncuoğlu *et al.,* 1984). The homologous nonapeptide AVP in birds is arginine vasotocin (AVT) (Acher *et al.,* 1970). Similarly to the function in mammal, AVT, a neurohypophyseal hormone in non-mammalian vertebrates, is involved in water balance in birds (Stallone and Braun, 1986). However, plasma levels of AVT increased in hypertonic solution and this response was further enhanced by naloxone injection as the doses increased (Saito *et al.,* 1999). Furthermore, i.c.v. injection of AVT increased body temperature (Tachibana *et al.,* 2004). These facts suggest that decreased body temperature induced by orally administered D-Asp could not be explained by increased AVT through the opioid system. Further investigations are needed to determine the relationships between D-Asp and possible factors to regulate body temperature. This finding raised the possibility that D-Asp may be a useful hypothermic agent at HT not only for layer chicks but also for broiler chicks. Importantly, D-Asp did not reduce food intake but caused to reduce rectal temperature in broiler chicks, which clearly indicate

that the body temperature reduction by D-Asp may be due to its specific action on central or peripheral thermoregulatory mechanisms in broiler chicks.

Evaluation of plasma metabolites in mammals and birds allow for the identification of metabolic alterations that are due to the effect of several factors, including pharmacological condition, age, physiological state, husbandry condition, and genetic type (Meluzzi *et al.,* 1992; Harr, 2002; Gayathri *et al.,* 2004; Juráni *et al.,* 2004; Alonso-Alvarez, 2005; Yanni *et al.,* 2005, 2010). In this study, we therefore determined the concentrations of glucose, TG, TCHO and TP to estimate whether the physiological condition of chicks was changed due to oral administration of L- or D-Asp. In Experiment 1, it was found that L-Asp did not influence any plasma metabolites studied. This finding confirmed to our previous report in layer chicks (Erwan *et al.,* 2014a). In addition, Tada *et al.* (2008) demonstrated that a subchronic oral administration of L-Asp with a dietary concentration of up to 5.0% had no influence on serum glucose in rats. Moreover, Delaney *et al.* (2008) and Karaman *et al.* (2011) also revealed that oral administration of L-Asp or N-acetyl-L-aspartic acid, an N-acetylated derivative of L-Asp, had no effect on TG, glucose, or TP. Thus, the present data corroborate those reported by others indicate that short-term or even long-term administration of L-Asp or its derivative may not change the plasma metabolites in chicks and rats.

Consistent with previous research (Erwan *et al.,* 2014a), plasma TG was found to be significantly decreased with the oral administration of D-Asp in the current study. It was further demonstrated that D-Asp decreased plasma TG in rats under free or restricted feeding conditions (Koyuncuoğlu *et al.,* 1982b). Shida *et al.* (1977) revealed that hypothermia resulted in decreasing plasma TG. It was further confirmed in this study that oral administration of D-Asp can increase glucose (Table 2) as observed in layer chicks (Erwan *et al.,* 2014a). Significant positive correlations between the dose of D-Asp and glucose clearly suggest that D-Asp might increase glucose. These findings are consistent with earlier report that revealed that hypothermia resulted in increasing

glucose (Shida *et al.,* 1977). In addition, Marley and Stephenson (1975) revealed that hypothermia was associated with increased blood glucose concentration in chicks. Therefore, the present results indicate that D-Asp induced hypothermia caused to increase energy demand in broiler chicks and thereby increased plasma glucose and decreased plasma TG.

In conclusion, oral administration of D-Asp, but not L-Asp, caused hypothermia in broiler chicks without affecting food intake and altered some plasma metabolites – namely, TG, and glucose. These results suggest the possibility of D-Asp as a potential hypothermic agent in chickens to mitigate heat-stress problems.

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Figure legends

Fig. 1. Effect of orally L-Asp administration on rectal temperatures in chicks during 120 min of the experimental period. The number of chicks used in each group ranged between 5 - 7. Values are means ± S.E.M.

Fig. 2. Effect of orally D-Asp administration on rectal temperatures in chicks during 120 min of the experimental period. The number of chicks used in each group ranged between 5 -7. Values are means ± S.E.M.

Table 1. Effects of oral administration of several doses of L-Asp on plasma metabolites in chicks

	L-Asp (mmol)			
Parameters	0	3.75	7.5	15
Glucose (mg/100 ml)	139 ± 11	127 ± 5	127 ± 4	125 ± 3
Total cholesterol (mg/100 ml)	192 ± 8	192 ± 9	181 ± 11	194 ± 13
Total protein (g/100 ml)	1.57 ± 0.1	1.54 ± 0.1	1.26 ± 0.1	1.54 ± 0.2
Triacylglycerol (mg/100 ml)	150 ± 11	157 ± 23	154 ± 14	140 ± 14

Values are means ± SEM. The number of samples used for analysis was 6-8.

Table 2. Effects of oral administration of several doses of D-Asp on plasma metabolites in chicks

	D-Asp (mmol)			
Parameters	0	3.75	7.5	15
Glucose (mg/100 ml)	124± 4[a]	131 ± 2[ab]	129 ± 3[ab]	140 ± 3[b]
Total cholesterol (mg/100 ml)	204 ± 5	210 ± 7	232 ± 22	200 ± 12
Total protein (g/100 ml)	1.67 ± 0.1	1.62± 0.1	1.63 ± 0.1	1.74 ± 0.1
Triacylglycerol (mg/100 ml)	147 ± 15[a]	107 ± 15[ab]	136 ± 9[a]	75 ± 8[b]

Means with different superscripts were significantly different at $P < 0.05$.

Values are means ± SEM. The number of samples used for analysis was 6-8.

Fig. 1

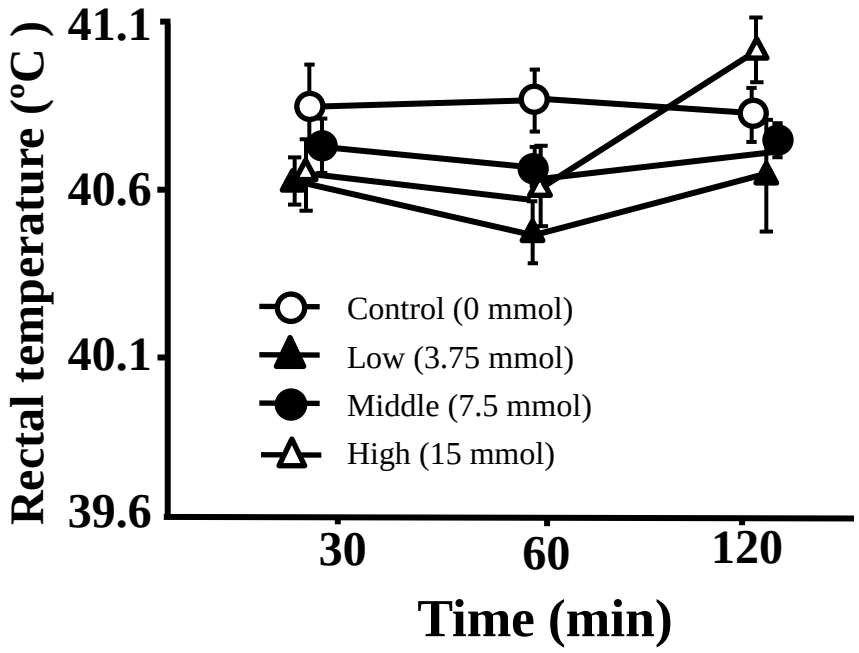
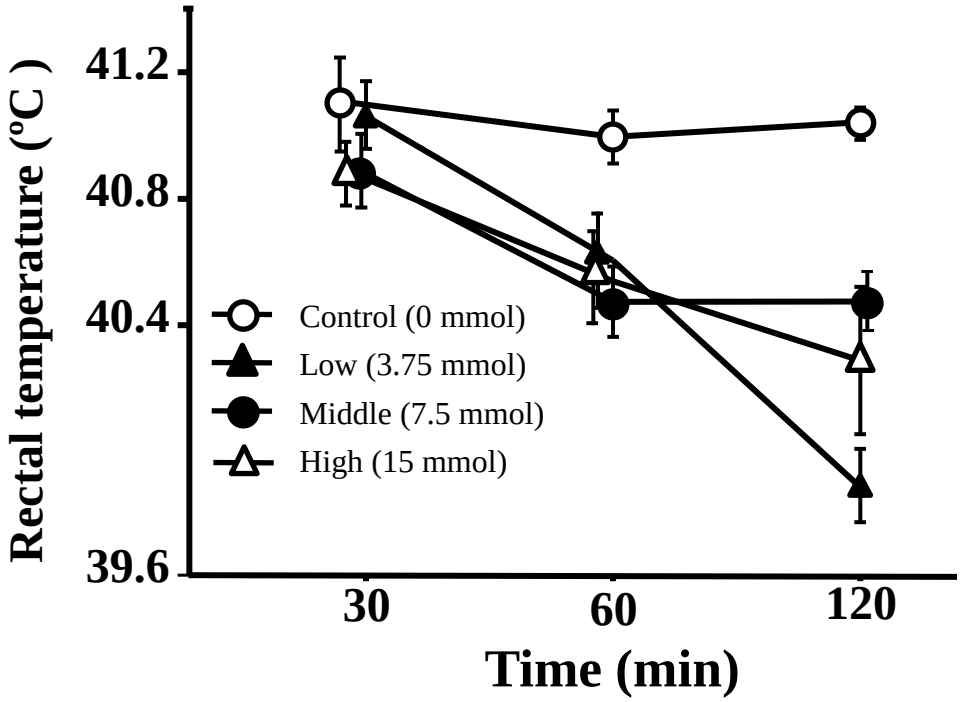

Rectal temperature (°C)
41.1
40.6
40.1
39.6
30
60
120
Time (min)
Control (0 mmol)
Low (3.75 mmol)
Middle (7.5 mmol)
High (15 mmol)

Fig. 2


Rectal temperature (°C)
41.2
40.8
40.4
39.6
30
60
120
Time (min)
Control (0 mmol)
Low (3.75 mmol)
Middle (7.5 mmol)
High (15 mmol)

To Authors

Although the title clearly states the emphasis of the study (viz. general physiological aspects), it was also to evaluate the effect or implication of L- and D-Asp on feed consumption or nutrient utilization. But, this information was minor in the discussion section. In addition, the number of bird per replicate was too small (only 1 although it had 4 replicates per treatment) and sampling was performed only one short time (after 120 minute). I am worried whether the statistical analysis would give accurate results, especially when interpreting data related to feed intake.

ABSTRACT

P2L3-5 : Suggest to add information of what specific physiological roles L- or D-Asp might affect (feed intake, energy metabolism, plasma metabolites, etc.)
: If there was study done with layer chicks then this last sentence need a revision.

P2L8-9 : Need to include statistical design.
: Need to mention the age of the chicks.
: Need to mention what samples were taken, sampling method, and time.
: Need to mention how you measured temperature of the rectum.

P2L10-11 : What do you mean by “we confirmed”? Do you mean “the study showed that ….?”

P2L19 : Suggest to write “rectal temperature” instead of “body temperature”.

INTRODUCTION:

P3L2 : Insert “-“between “induced and heat” to make this sentence meaningful.

P3L8 : Better not to use abbreviation (e.g. HT) to start a sentence.

P3L17 : Remove ”,” punctuation.

P4L6 : remove “but”.

P4L7-9 : Suggest to remove the last sentence.

P4L25 : You did not determine the dose of significant, so it is suggested to use “evaluate” instead of “determine”.

MATERIALS AND METHODS

P5L3 : Too many hypens used, suggest to use “One-day-old broiler chicks”.

P5L5-6 : Is this the dimension of cage for the all chicks?
: Add information of ME and protein content of the diet.

P5L9 : How many birds did you keep initially in the wooden cage?
: What is the dimension of the cage for individual bird?

P5L14 : Suggest to add “both of” to start the sentence.

P5L18-21 : It seems that you had 4 groups or treatments in each experiment, and each treatment consisted of 8 replicates with one bird each? Was it enough for statistical analysis?

P6L5-6 : replace “food” with “feed” (and also in other sentences).

P6L9 : How long was the probe in the cloaca?

P612 : Where did you take the blood from?
: What was the g-force (centrifugal force) of the centrifuge?

RESULTS

P7L6 : Suggest to display feed intake data because it was one of the objectives of the study as mention in the Introduction (P4L24-26)

P7L7 : No need to mention the probability value if the data were not significant.

P7L15 : see suggestion in P7L6.

P7L25 : Remove "," (similarly with P8L2).

DISCUSSION

P9L7 : Is there any information of what potential effect might be observed due to different enantiomers of aspartate (L vs. D)?

P9L15-18 : Assuming broiler has greater rate of feed intake and metabolism, why the effect of
: D-Asp was significant in layer but not in broiler?

P9L22-24 : Need clarification as questioned in P9L15-18

P10L3-7 : Suggest to remove (or summarized in Introduction).

P10L7-9 : No need to repeat (already mentioned in the Introduction).

P10L11 : remove "to".

P10L17-18 : Suggest to remove "short term or even long term" to avoid a generalized conclusion. It is suggested to add "especially in short term administration" after chicks (remove rats).

P10L21-23 : Suggest to revise and combine the two sentences. For instance: Studies with rat (Koyuncuoglu et al.) and chick? (Shida et al) clearly showed the effect of …."

P10L24 : Past tense is suggested to use.

P10L25-26 : Make sure that R^2 is high enough to say correlation between 2 variables, otherwise say it softly.

P11L3 : Need to correct the grammar.

P11L6 : Replace "caused" with "could cause".

TABLES AND FIGURES

P19L1 and 8 : Add information of which experiment each figure refer to (check the unit, mmol/kg; need to put R^2)
: Similarly please check the Tables (insert "in the same row" before "were")

February 21, 2016

Reviewer

Response to the Reviewer

In taking into account valuable comments offered by the Reviewers, the manuscript entitled ' Orally Administered D-Aspartate Depresses Rectal Temperature and Alters Plasma Triacylglycerol and Glucose Concentrations in Broiler Chicks ' has been carefully revised and rewritten. Specific changes to improve the manuscript according to the comments of the Reviewers are as follows:

Reviewer # 1*:* This reviewer is favorable and has given additional important comments.
To Authors
Although the title clearly states the emphasis of the study (viz. general physiological aspects), it was also to evaluate the effect or implication of L- and D-Asp on feed consumption or nutrient utilization. But, this information was minor in the discussion section. In addition, the number of bird per replicate was too small (only 1 although it had 4 replicates per treatment) and sampling was performed only one short time (after 120 minute). I am worried whether the statistical analysis would give accurate results, especially when interpreting data related to feed intake.

Comments on Abstract

 # Point 1. Suggest to add information of what specific physiological roles L- or D-Asp might affect (feed intake, energy metabolism, plasma metabolites, etc.)". : If there was study done with layer chicks then this last sentence need a revision.

Response: In line with the suggestion, we have modified the sentence (Please see the revised MS, Page 2, lines 3-5).

 # Point 2: Line 8-9 *Need to include statistical design: Need to mention the age of the chicks.: Need to mention what samples were taken, sampling method, and time.: Need to mention how you measured temperature of the rectum.*

Response: According to this comment, we have added the following sentences in the Abstract section "A repeated-measures two-way ANOVA was applied for the analysis of food intake in both Experiments 1 and 2. Plasma metabolites were statistically analyzed by one-way ANOVA and regression equations. Broiler chicks (5 days old) were orally administered with different doses (0, 3.75, 7.5 and 15 mmol/kg body weight) of L- or D-Asp. At 120 min after administration of L- or D-Asp, the blood was immediately collected through the jugular vein into heparinized tubes and centrifuged. The rectal temperature of chicks was measured using a digital thermometer with an accuracy of ± 0.1°C, by inserting the thermistor probe in the cloaca to a depth of 2 cm". (Please see the revised MS, Page 2, lines 9-17).

 # Point 3. What do you mean by "we confirmed"? Do you mean "the study showed that ....?"

Response: According to this comment, we have change `*we confirmed*` *to " the study showed that "* in the sentence (Page 2, line 17).

 # Point 4. Suggest to write "rectal temperature" instead of "body temperature".

Response: In line with the suggestion, we have rewritten the sentence (Please see the revised MS, Page 2, line 26

Comments on Introduction

Point # 1: *Insert "-"between "induced and heat" to make this sentence meaningful.*

Response: In line with the suggestion, we insert "-"between "induced and heat" (Please see the revised MS, Page 3, line 2)

Point # 2: *Better not to use abbreviation (e.g. HT) to start a sentence.*

Response: In line with the suggestion, we have modified the sentence (Please see the revised MS, Page 3, line 8)

Point # 3: *Remove "," punctuation.*

Response: In line with the suggestion, we have removed "," punctuation in the sentence (Please see the revised MS, Page 3, line 17)

Point # 4: *remove "but".*

Response: In line with the suggestion, we have deleted "but" (Please see the revised MS, Page 4, line 6)

Point # 5 *Introduction: Page 4 Line 25 You did not determine the dose of significant, so it is suggested to use "evaluate"*

Response: In line with the suggestion, we have modified the sentence

Point # 6 *Materials and Method Page 5 Line 3 : Too many hypens used, suggest to use "One-day-old broiler chicks".*

Response: In line with the suggestion, we have modified the sentence

Point # 7 *Materials and Method: P5L5-6 : Is this the dimension of cage for the all chicks?: Add information of ME and protein content of the diet.*

Response: No, we initially kept 20-25 chick in a cage from 1 to 4 days old. We have two cages to kept all chicks. Then chicks were placed in individual cages (floor space: 20 cm × 25 cm; height:

25 cm) 24 h before the start of the experiment. In line with the suggestion, we have modified the sentence

Point # 8 *Materials and Method Page 5 Line 9 : How many birds did you keep initially in the wooden cage?: What is the dimension of the cage for individual bird?*

Response: We initially kept 20-25 birds in the wooden cage. *The dimension* for individual was 20 cm x 25 cm; height 25 cm. So we have revised the sentence

Point # 9 *Materials and Method P5L14 : Suggest to add "both of" to start the sentence.*

Response: In line with the suggestion, we have modified the sentence

Point # 10 *Materials and Method Page 5 Line 18-21 : It seems that you had 4 groups or treatments in each experiment, and each treatment consisted of 8 replicates with one bird each? Was it enough for statistical analysis?*

Response: We sure those replications were enough for statistical analysis. We used to same replications in experimental chicks based on our previous reports (Erwan et al., 2013a; Erwan et al., 2013b; Erwan et al., 2014; Ito et al., 2014)

Point # 11 *Materials and Method P6L5-6 : replace "food" with "feed" (and also in other sentences).*

Response: In line with the suggestion, we have modified the sentence

Point # 12 *Materials and Method P6L9 : How long was the probe in the cloaca?*

Response: We did about 1-5 seconds. The most important is the dept about 1-2 cm and stop until the number of degree appeared constant/not changes.

Point # 13 *Materials and Method P612 : Where did you take the blood from?: What was the g-force (centrifugal force) of the centrifuge?*

Response: We have collected blood through the jugular vein. The the g-force (centrifugal force) of the centrifuge was rpm/rct.

Point # 14 *RESULTS Page 7 Line 6 : Suggest to display feed intake data because it was one of the objectives of the study as mention in the Introduction (P4L24-26*)

Response: In line with the suggestion, we have shown feed intake data in the sentence (Please see the revised MS, Page 4, line 6)

Point # 15 RESULTS P7L7 : *No need to mention the probability value if the data were not*

significant.

Response: In line with the suggestion, we have deleted the probability value (Please see the revised MS, Page 4, line 6)

Point # 16 *Results Page 7 Line 15 : see suggestion in P7L6.*

Response: In line with the suggestion, we have shown feed intake data in the sentence (Please see the revised MS, Page

Point # 17 *Results Page 7 Line 25 : Remove "," (similarly with P8L2).*

Response: In line with the suggestion, we have removed *"," in* the sentence

Point # 18 *DISCUSSION P9L7 : Is there any information of what potential effect might be observed due to different enantiomers of aspartate (L vs. D)?*

Response: It seems to be an established fact that the metabolism and utilization of L- and D-Asp is different. For instance, we revealed the differences between the effect of oral and i.c.v. administration of L-Asp and D-Asp on stress response, food intake and regulation of body temperature. In addition, the mechanism of stress attenuating function of L- and D-Asp possibly occurs through different receptors (Erwan et al., 2012, 2013, 2014). Koyuncuoğlu and Berkman (1982) revealed that in rats, the concomitant oral administration of L-Asp seemed to antagonize the effect of D-Asp. In addition, previous report revealed that the injection of D-Asp but not L-Asp released arginine vasotocin (AVT) in rats (Koyuncuoğlu et al., 1984). Hence, these information raised the possibility of having different mechanism between the two isomers.

Point # 19 *DISCUSSION P9L15-18 : Assuming broiler has greater rate of feed intake and metabolism, why the effect of: D-Asp was significant in layer but not in broiler?*

Response:
Genetic selection has altered not only performance, but also temperament in domestic chickens (Saito *et al.,* 2005). Furuse *et al.* (2007) suggested that layers have larger containing higher levels of anorexigenic neuropeptides. Numerous reports have proven the existence of strain differences especially between meat-type chickens and layer-type chickens in the stress response. For instance, both locomotive activities and plasma corticosterone concentrations were higher in layer than in broiler chicks after isolation-associated stress (Saito *et al.,* 2005). Our previous finding also found that oral D-Asp clearly increased D-Asp levels in the plasma and diencephalon suggested that D-Asp may act as an anorexigenic factor in the diencephalon (Erwan *et al.,* 2013). Although we did not measure the D-Asp concentration in current experiment but we speculate that this alteration would not be in broiler. The reason underlying this difference is not clear; however, it may be accounted for by relative adrenal weight and cell function. In other word we suggest that broilers have either greater capability to adapt to a novel milieu or are much less sensitive to changes in environment compare to layers.

Point # 20 *DISCUSSION P9L22-24 : Need clarification as questioned in P9L15-18*

Response:,Instead of similar to point 19 explanation and based on our present result it is indicate that the body temperature reduction by D-Asp may be due to the same of its specific action on central or peripheral thermoregulatory mechanisms in broiler chicks as well as in layer chick.

Point # 21 *DISCUSSION P10L3-7 : Suggest to remove (or summarized in Introduction).*

Response: According to this important comment, we have removed

Point # 22 *DISCUSSION P10L7-9 : No need to repeat (already mentioned in the Introduction)*

Response: In line with the suggestion, we have deleted those sentences (please see the revised MS, Page 9 line 7)

.

Point # 23 *DISCUSSION P10L11 : remove "to".*

Response: Response: In line with the suggestion, we have deleted *"to".* (Please see the revised MS, Page, 10 line 6)

Point # 24 *DISCUSSION P10L17-18 : Suggest to remove "short term or even long term" to avoid a generalized conclusion. It is suggested to add "especially in short term administration" after chicks (remove rats).*

Response: In line with the suggestion, we have modified the sentence (Please see the revised MS, Page, 10 line 14)

Point # 25 DISCUSSION P10L21-23 : Suggest to revise and combine the two sentences. For instance: Studies with rat (Koyuncuoglu et al.) and chick? (Shida et al) clearly showed the effect of …."

Response: In line with the suggestion, we have modified the sentence (Please see the revised MS, Page, 10 lines 17-18)

Point # 26 *DISCUSSION P10L24 : Past tense is suggested to use*

Response: In line with the suggestion, we have modified the sentence (Please see the revised MS, Page, 10 line 19)

Point # 27 *DISCUSSION P10L25-26 : Make sure that R2 is high enough to say correlation between 2 variables, otherwise say it softly.*

Response: In line with the suggestion, we have modified the sentence (Please see the revised MS, Page, 10 line 20)

Point # 28 *P11L3 : Need to correct the grammar.*

Response: In line with the suggestion, we have modified the sentence (Please see the revised MS, Page, 10 line 27)

Point # 29 *DISCUSSION P11L6 : Replace "caused" with "could cause".*

Response: In line with the suggestion, we have *replace "caused" with "could cause"* in the sentence (Please see the revised MS, Page, 11 line 1)

Point # 30 *TABLES AND FIGURES P19L1 and 8 : Add information of which experiment each figure refer to (check the unit, mmol/kg; need to put R2)*

Response: In line with the suggestion, we have modified the sentence (Please see the revised MS, Page, 19 lines 2 and 6)

Point # 31: Similarly please check the Tables (insert "in the same row" before "were")

Response: In line with the suggestion, we have insert "in the same row" before "were" the sentence

Reviewer 2

Major comments are follows:
Your results showed that D-Asp plays a role of decreasing body temperature in chickens and inhibiting heat stress induced high ambient temperature. How do you think this result apply for a field of poultry farm.

Response: Summer heat stress especially in chicken is becoming more unbearable in many tropical and subtropical countries. Of course this result has benefit and may potentiate to be implemented in field of poultry farm. However, nutritional manipulation with low cost involvement is a common approach in poultry industry. Hence, it is needed to find out some sources which cheap and easy to use for instance from agriculture by-products which rich of D-Asp.

Point # 1: *In the case of D-Asp including the diet for chickens, is it conceivable that D-Asp might inhibit incorporation of L-Asp?*

Response: Yes, some studies of D-Asp including the diet in chicks (Maruyama et al., 1967) and rats (Koyuncuoğlu et al., 1982b; Koyuncuoğlu and Berkman, 1982) revealed that D-Asp appeared to inhibit the effect of L-Asp.

Point # 2: *In this study, blood samples were collected at 120 min after oral administration. Has it been cleared that the metabolic rate and absorptive rate of D-Asp? What study did base on your experimental plan.*

Response: We have revealed some plasma metabolite changes after 120 min oral administration of D-Asp in chicks (Erwan et al., 2014) indicated there were metabolic rate and absorptive rate of D-Asp in blood.

Point # 3: *In the result of plasma metabolites oral administration of D-Asp decreased the concentration of glucose and triglyceride. Is it possible that long-term intake of D-Asp leads to a bad influence for chickens?*

Response: First, we clarified that oral administration of D-Asp decreased the concentration of triglyceride but not glucose. It seems that if long-term intake of D-Asp leads to a bad influence for chicken. To prevent this negative effect, it is needed to find out the optimum level/concentration which suitable to have benefit for alleviate heat stress while not change the metabolite of plasma especially triglyceride.

Point # 4*: Your study showed that the range of glucose concentration in the plasma was from 120 to 140. Glucose level was seemed to a little lower than other articles. For example, Lu et al., (2007. Pout. Sci. 86, 673-683) reported that glucose concentration in the plasma of 5 days of chicks were over 200 mg/100 mL. How do you describe these differences?*

Response: We are not sure why this was happen in glucose concentration. We can compare the data within experiments but not between experiments. We have also have experiences when determined the the basal values of dopamine (DA) in in the diencephalon or telencephalonin 5-day of chicks. For instance, Shiraishi *et al*. (2010) reported that the basal content of DA in the diencephalon in chicks was 283 pg/mg and 320 pg/mg, meanwhile (Erwan *et al*., 2012; Katayama *et al*., 2010) determined the basal content of DA was 1,487 pg/mg and 1,546 pg/g, respectively. In the telencephalon the basal value of DA was 376 pg/mg (Saito *et al*., 2004), and 545 pg/mg (Erwan *et al*., 2012), meanwhile Katayama *et al.* (2010) and Katayama *et al.* (2011) reported that basal value of DA was 2,932 pg/g and 432.8 pg/mg, respectively. However, the influence seems minimal when focusing on the difference in effects between L- or D-Asp on rectal temperature and food intake which was the major concern in the present studies

The minor comments.

Point # 5 *Page 5, line 7: The percentage of crude protein and metabolizable energy in the commercial starter diet should be shown.*

Response: In line with the suggestion, we have added those information in the sentence

Point # 6 *Page 5, line 9: The average of body weight of chickens should be shown.*
Page 6, line 13: "rpm" should be shown at "x g"

Response: In line with the suggestion, we have changed `rpm` to ` x g`.

Point # 7: *In the part of "Result", Figs and Tables should be indicated in the sentence*

Response: In line with the suggestion, we have modified the sentence (Please see the revised MS, Page, 7 lines 6, 8 16, 17 and 19).

To Authors

They are still few corrections suggested (see below).

ABSTRACT

P2L12-17 : These sentences should come prior to statement of statistical analysis.

MATERIALS AND METHODS

P6L13-14 : Suggest to revise to “Blood Collection and Analysis of Plasma Metabolites”.
: Then, start the paragraph with the sentence from L2-4 (remove this sentence from their original place)
: Need to explain here how many birds you took blood from in each exp unit.

P6L16 : Remove one of the double brackets at the end of “TP”

RESULTS

P7L8-9 : No need to mention the F and probability value if the data were not significant.

P7L19 : Same suggestion (and to all the subsequent related sentence) as above.

P8L3-4 : When presenting a regression equation in the text, no need to put the SE, just the equation and R^2 value.

DISCUSSION

P9L19 : insert “)” after 1982.

P9L20 : Suggest to revise to “…we found….”

P9L25 : Revise to “Previous research findings revealed that ….”.

September 23, 2016
Reviewer

State Islamic University of Sultan Syarif Kasim Riau

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Dr. Kuniko Yasue
Editor-in-Chief
THE JOURNAL OF POULTRY SCIENCE

December 20, 2016

Dear Dr. Kuniko Yasue,

Kindly please lest us to submit our revision of manuscript entitled " Orally Administered D-Aspartate Depresses Rectal Temperature and Alters Plasma Triacylglycerol and Glucose Concentrations in Broiler Chicks to Journal of Poultry Science".

Please find our electronically-submitted Manuscript

I sincerely hope that the manuscript is acceptable for publication.

Thank you very much in advance for your consideration of the manuscript.

Sincerely yours,

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Response to the Reviewer # 1

JPSA-2016-0010R1

In taking into account the further comments offered by the Reviewer # 1, the manuscript entitled " Orally Administered D-Aspartate Depresses Rectal Temperature and Alters Plasma Triacylglycerol and Glucose Concentrations in Broiler Chicks" has been carefully revised and rewritten. Specific changes to improve the manuscript according to the comments of the Reviewers are as follows:

Comment on ABSTRACT

P2L12-17: These sentences should come prior to statement of statistical analysis.
Response: Changed as per the comment.

Comment on MATERIALS AND METHODS

P6L13-14 : Suggest to revise to "Blood Collection and Analysis of Plasma Metabolites".
: Then, start the paragraph with the sentence from L2-4 (remove this sentence from their original place)
: Need to explain here how many birds you took blood from in each exp unit.
Response: We have changed accordingly. Please the revised MS.

P6L16 : Remove one of the double brackets at the end of "TP"
Response: As last one of the double bracket is a part of the first bracket started after "The plasma metabolites (TG,…..", we have not deleted the double bracket.

Comments on RESULTS

P7L8-9 : No need to mention the F and probability value if the data were not significant.
P7L19 : Same suggestion (and to all the subsequent related sentence) as above.
Response: We have removed the F values as per the comment.

P8L3-4 : When presenting a regression equation in the text, no need to put the SE, just the equation and R2 value.

Response: Done accordingly.

Comments on DISCUSSION

P9L19 : insert ")" after 1982.

P9L20 : Suggest to revise to "…we found…."

P9L25 : Revise to "Previous research findings revealed that ….".

Response: We have revised as per the comments. Please see the revised MS.

The Journal of Poultry Science

Decision Letter (JPSA-2016-0010.R2)

From: eic-jps@jpn-psa.jp

To: erwan_edi@yahoo.com

CC: jpsa-s@naro.affrc.go.jp

Subject: The Journal of Poultry Science - Manuscript ID JPSA-2016-0010.R2

Body: 21-Dec-2016
Manuscript ID JPSA-2016-0010.R2
Orally Administered D-Aspartate Depresses Rectal Temperature and Alters Plasma Triacylglycerol and Glucose Concentrations in Broiler Chicks

Dear Dr. Erwan:

Thank you very much for your contribution to the Journal of Poultry Science.

I am pleased to inform you that the peer review of your manuscript is now completed, and it can be recommended for publication at the Journal of Poultry Science.

I would like to inform you, however, that we have introduced a new policy for non-member contributor that the "official letter of acceptance" will not be issued until the payment of page-charge has been confirmed. For detail, please refer to " Instruction for Authors" which became in effect on July 25, 2014,

The quote of the page charge required to publish your contribution will be sent to you soon from the editorial office.

Thank you very much for your understanding and cooperation.

Sincerely yours,

Dr. Atsushi Tajima
Editor in Chief, The Journal of Poultry Science
eic-jps@jpn-psa.jp

Dear Dr. Erwan,

On behalf of the editors of Journal of Poultry Science, we are pleased to inform you that your manuscript entitled "Orally administered D-aspartate depresses rectal temperature and alters plasma triacylglycerol and glucose concentrations in broiler chicks (by JPSA-2016-0010R2)" has been accepted for publication.

Thank you for submitting your manuscript for consideration in Journal of Poultry Science.

Yours sincerely,

Tetsuya Tachibana

Editor
Journal of Poultry Science

****
Editorial office
Japan Poultry Science Association
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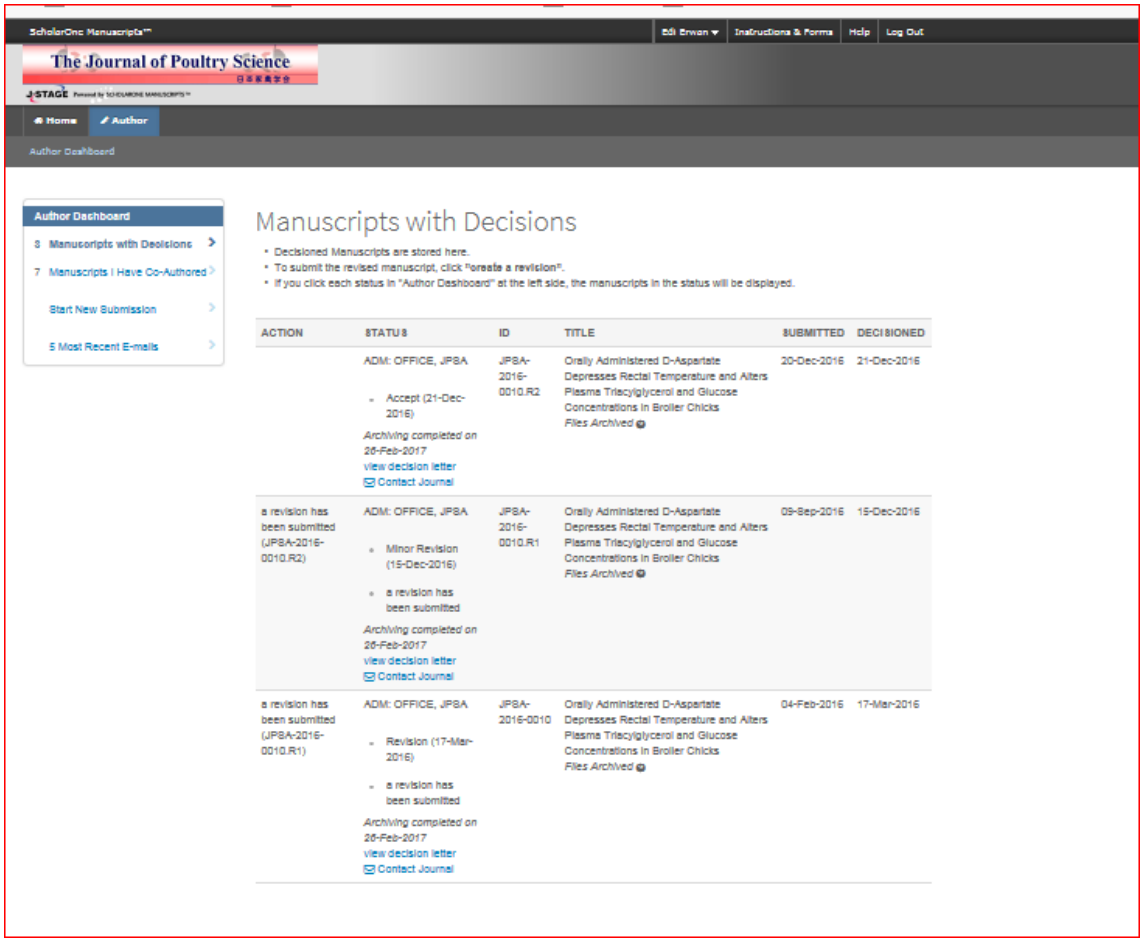
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